Reference: *Biol. Bull.* **197:** 319–331. (December 1999)

Larval Ontogenetic Stages of *Chaetopterus*: Developmental Heterochrony in the Evolution of Chaetopterid Polychaetes

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Abstract. Seven post-gastrulation larval stages are described for the sedentary polychaete *Chaetopterus*. Analysis of larval anatomy and morphology through ontogeny reveals significant differences in the temporal sequence of segmentation, and in the character of segments formed, from the typical embryological pattern described for other polychaete families, such as nereidids or spionids. When compared in alternative phylogenetic schemes, these differences represent significant developmental heterochrony, among other evolutionary transitions, which has arisen in the chaetopterid lineage. The heterochrony is correlated with the extreme morphological regionalization along the anterior-posterior body axis, a feature that is also characteristic of chaetopterids.

Introduction

The peculiar morphology and lifestyle of the sedentary polychaetes of the genus *Chaetopterus* have long attracted the interest of a range of biologists. *Chaetopterus* is unique among annelids in the degree of morphological differentiation of segments along the body axis. However, the larval ontogeny of representatives of the common North American species complex has never been completely described. This work is meant to be a basis for comparative studies of molecular development in this heteronomously segmented worm. The phylogenetic position of *Chaetopterus* and its implications for variation in developmental programs is also discussed.

Adult chaetopterids live in mucus-lined U-shaped tubes, either partly buried in the sediment or attached to hard objects such as stones or corals. The tubes are usually paperlike, hence the common name *parchment worm*. The worm feeds by pumping seawater through its tube with modified parapodial "fans," catching suspended organisms in a mucous net that it then rolls up, using the accessory feeding organ; sends to the mouth, *via* the mid-dorsal ciliary groove; and ingests (MacGinitie, 1939). The body form and parapodia are highly modified in relation to members of other polychaete families to enable this unique feeding behavior. The chaetopterids have generally been considered to be related to the spioniform families, but their unique and very complex external morphology sets them apart from other polychaetes (Fauchald and Rouse, 1997). The purpose of studying *Chaetopterus* in the present context is as a basis for comparative evolutionary embryology aimed at elucidating the developmental sources of morphological innovation.

The genus *Chaetopterus* is a species complex sometimes mistakenly described as monotypic (Petersen, 1984a, b). The species we have used is that described by Enders (1909) as *Chaetopterus variopedatus*; however, it is different from the type species *C. variopedatus* Renier, 1804. Unfortunately, the type specimen of *C. variopedatus* has been lost, making comparison uncertain (M. E. Petersen, Copenhagen Museum, personal correspondence). We will refer to the animal we describe, commonly available in Woods Hole, Massachusetts and Beaufort, North Carolina, as *Chae-*

Received 28 April 1999; accepted 5 August 1999.

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topterus. A more definitive name awaits a complete revision of the genus (M. E. Petersen, in preparation).

Chaetopterus was an important model for classical studies of early embryogenesis (Mead, 1897; Lillie, 1906). The accessibility of large numbers of gametes in *Chaetopterus* has made it a model organism for the cell biology and biochemistry of eggs and sperm (*e.g.*, Inoué *et al.*, 1974; Jeffery, 1985; Eckberg and Anderson, 1995). Since the gametes are easily fertilized *in vitro*, *Chaetopterus* lends itself to studies of early cleavage and embryogenesis (*e.g.*, Mead, 1897; Henry, 1986, 1989; Henry and Martindale, 1987).

Several turn-of-the-century papers describe the anatomy and life history of *Chaetopterus* in some detail (Joyeux-Laffuie, 1890; Béraneck, 1894; Enders, 1909). Joyeux-Laffuie established a scheme for numbering segments (not a trivial matter) in the adult worm that has been followed by subsequent authors. Cazaux (1965) has the most complete description of external larval morphology in the literature, although his findings differ from ours in some respects (see below). Bonch-Bruevich and Malakhov (1987) describe the internal anatomy of the 48-h larva on the basis of transmission electron micrography. Detailed early embryogenesis was traced by Mead (1897) and more recently by Malakhov (1984) and Henry (1986) through 72 h of development; this paper thus concentrates on postgastrulation larval ontogeny through metamorphosis. We also present the first photographic documentation of larval ontogeny and propose a scheme for numbering the larval stages. The ontogeny is then analyzed in a phylogenetic context to propose the occurrence of a novel developmental heterochrony in the chaetopterid lineage.

Materials and Methods

Larval culture. Methods for obtaining and fertilizing gametes are described by Henry (1986) and Eckberg and Hill (1996). Specific aspects of methods used in this study are described in Irvine and Martindale (1999a). Larvae through stage L4 were photographed using DIC (differential interference contrast) optics on a Zeiss Axioplan microscope. Later stages were imaged on a Wild M8 stereo microscope using an Optronics DI-750 3-chip CCD camera and digitized with a PDI frame grabber on a Macintosh computer.

Staining and sectioning. Larvae were fixed in 4% formaldehyde in filtered artificial seawater for 30 min., washed, and stored in PTw (phosphate buffered saline + 0.1% Tween-20) at 4°C. They were dehydrated in a 25%, 50%, 70%, and 90% ethanol series and embedded in JB-4 medium (Polysciences) in accordance with the manufacturer's instructions. During the dehydration, some of the animals were stained with 1% alcian blue in 70% ethanol for 20–30 min. Serial sections (4 μm thick) were cut on a Sorvall MT2-B ultramicrotome using diamond knives. Sections were stained with Harris's hematoxylin and 0.1% eosin Y and mounted in 70% glycerol.

β-tubulin RNA probe. A 780-bp fragment of the *β-tubulin* gene was amplified by polymerase chain reaction using degenerate oligonucleotide primers from a larval *Chaetopterus sp.* cDNA library (details available on request). Two primers were used:

b-tub.for: 5′-TGGGCNAARGGNCAYTA
b-tub.rev: 5′-ATNCCYTCNCCNGTRTAC

Amplicon termini were prepared for cloning with T4 DNA polymerase and T4 polynucleotide kinase (New England Biolabs) and ligated into pMOB vector cut with SmaI. The resulting ligation was transformed into *E. coli* by electroporation and sequenced on both strands. The DNA sequence has been deposited in GenBank under accession number AF140551. Sense and antisense digoxygenin-labeled riboprobes were transcribed from the plasmid template using the MegaScript kit (Ambion) according to the manufacturer's instructions, except for nucleotide concentrations. These were 7.5 m*M* GTP, ATP, and CTP, 6.35 m*M* unlabeled UTP, and 1.15 m*M* digoxygenin-11-UTP (Boehringer-Mannheim).

In situ *hybridization.* A detailed protocol is available on request—the following is a brief description of our procedure. Larvae were fixed for 20 min in buffered 4% formaldehyde at 50°C and prepared for hybridization by treatment with chitinase and acetic anhydride. The specimens were then incubated in a detergent solution (1% sodium dodecyl sulfate (SDS); 0.5% Tween-20; 50 m*M* Tris-HCl, pH 7.5; 1 m*M* EDTA, pH 8; 150 m*M* NaCl) for 1 h. Hybridization was done overnight at 65°–69°C with 0.1 μg/ml of digoxygenin-labeled probe in RNA-Hybe buffer (50% formamide; 5× saline sodium citrate, pH 4.5; 1% SDS; 0.1% Tween-20; 50 μg/ml heparin; 50 μg/ml yeast tRNA, 100 μg/ml sonicated salmon sperm DNA). The washed larvae were incubated in alkaline phosphatase conjugated sheep anti-digoxygenin Fab antibody (Boehringer-Mannheim), and the hybridization signal was developed with B-M purple chromogenic substrate (Boehringer-Mannheim), postfixed in 4% formaldehyde for 15 min, and cleared in 70% glycerol.

Results

Adult body plan

The adult worm consists of three functionally distinct body regions called, from anterior to posterior, regions A, B, and C (Crossland, 1904; Bhaud *et al.*, 1994) (Fig. 1). Region A is composed of the morphologically fused presegmental prostomium and peristomium along with the first nine setigers (A1–A9). A setiger is a body segment bearing setae (sometimes spelled *chaetae*). The prostomium bears

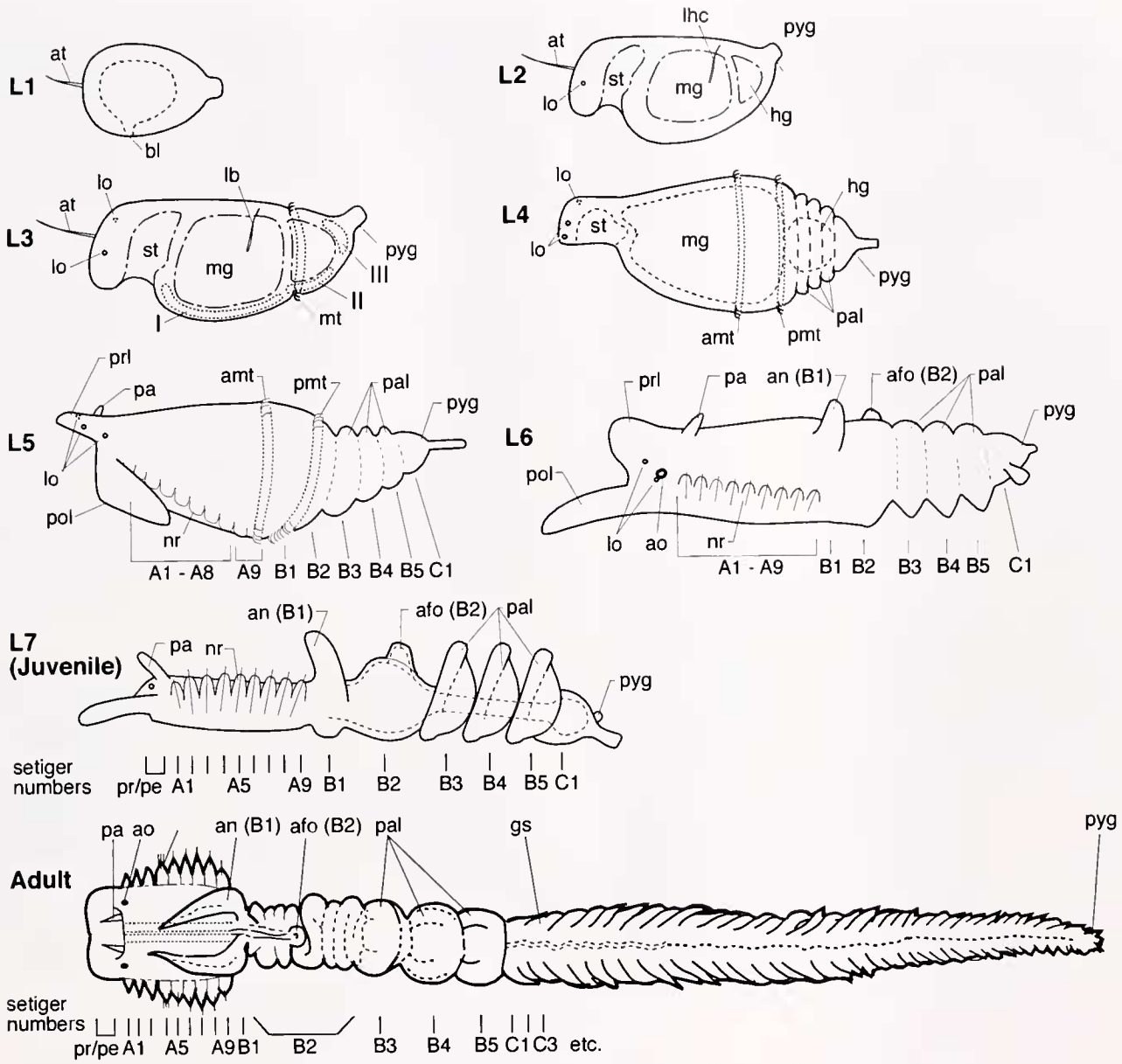

Figure 1. Diagrammatic views of *Chaetopterus* from larval stage L1 through adult, showing relationships between larval and adult structures. Larvae are in lateral view with anterior to the left and ventral towards the bottom of the page; the adult is in dorsal view, anterior to the left. The setiger numbering scheme is correlated below the drawings of stages L5, L6, L7, and the adult. Setiger numbering scheme follows Crossland (1904) and Bhaud *et al.* (1994). I, II, III, anterior, middle, and posterior larval trunk coeloms respectively; afo, accessory feeding organ; amt, anterior mesotroch; an, aliform notopodium; ao, adult ocellus; at, apical tuft; bl, blastopore; gs, gametogenic segments; hg, hindgut; lb, lateral bristle; lhc, lateral hooked cilia; lo, larval ocellus; mg, midgut; mt, mesotroch; nr, notopodial rudiment; pa, palp; pal, palette; pmt, posterior mesotroch; pyg, pygidium.

one pair of lateral ocelli, and the peristomium has a pair of small grooved palps. The setigerous region of region A is flattened dorsoventrally and bears only notopodia—except for setiger A9, which also has neuropodial uncini. Notopodia and neuropodia are the dorsal and ventral rami, respectively, of the parapodia. The notopodium of setiger A4 is modified and bears a bundle of dark spines, although this morphology did not appear in the postmetamorphic stages examined in this study. The slightly convex muscular ventral surface of region A is termed the *plastron*.

The middle tagma, region B, has five specialized segments that enable the peculiar suspension-feeding behavior.

Setiger B1 has dorsally extended aliform notopodia, which secrete and support a mucous net. This net is gathered by the accessory feeding organ of setiger B2, which is located dorsal to a convoluted portion of the gut. From this organ, a ciliated groove on the dorsal midline carries food particles anteriorly to the mouth. Setiger B2 is much longer than other segments, but derives from a single larval segment, as demonstrated below. The posterior three segments of this region consist of highly modified neuropodia forming fans termed *palettes* that pump water through the animal's tube by a metachronal, rhythmic movement (MacGinitie, 1939; Barnes, 1965). Each of the five segments in this middle region has a neuropodial torus bearing uncinal plates. Tori are flattened ridgelike parapodia, and uncini are dentate deeply imbedded setae with platelike bases (Fauchald, 1977).

The posterior tagma (region C) has an indeterminate number of gametogenic segments of a uniform morphology, tapering in size towards the posterior. Each segment bears a crescent-shaped notopodium and bilobed neuropodium. The small pygidium bears two pairs of cirri surrounding the anus.

Larval stages

The following stage-numbering system begins with the earliest pelagic larva. Although embryonic development prior to gastrulation and production of the swimming larva have been described (Malakhov, 1984; Henry, 1986), no complete staging scheme has been previously devised for *Chaetopterus* larvae. Developmental times are postfertilization ages based on laboratory culture at room temperature of 21°–23°C. Sizes listed are in micrometers, anterior-posterior length (excluding apical tuft and posterior papilla) by maximum lateral width (excluding setae). The numbering of segments is as shown in Figure 1.

Stage L1—18–36 hours (Fig. 2a):
Protrochophore
Size: 125 × 100 μm
Ciliary structures: apical tuft present; one pair of lateral hooked cilia; trochal band absent
Ocelli: absent
References: Henry (1986) fig. 3a; Cazaux (1965) plt. 1, fig. 4

The initial swimming gastrula, or protrochophore, is completely ciliated but lacks any trochal band. An apical cilia tuft about 50 μm in length is present. The gut has not achieved its functional form and is still filled with the progeny of the yolky macromeres. A bilateral pair of tufts of diffuse hooked cilia, termed *lateral hooked cilia* (Henry, 1986), form during this stage. At no time is a prototroch typical of early polychaete trochophore larvae formed (Henry, 1986; Eckberg and Hill, 1996).

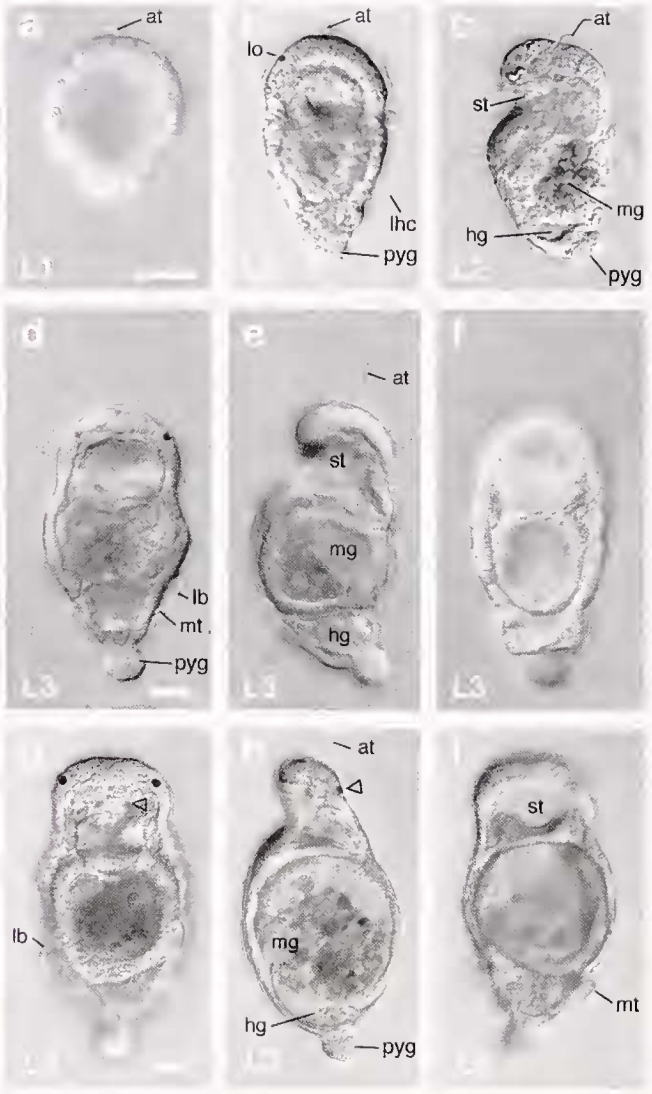

Figure 2. Larvae, stages L1 through L3. Anterior is toward the top of the page in all plates, unless noted. (a) Stage L1 protrochophore at 24 h. (b, c) Stage L2 metatrochophores at 48 h in ventral and left-lateral views respectively. (d–f) Early stage L3 larvae at 4 days in dorsal, lateral, and ventral views respectively. (g–i) Late stage L3 larvae at 18 days in dorsal, left-lateral, and ventral views respectively. Medial pair of eyes is slightly out of focus in (g) and at dorsal surface in (h) (open arrowheads). Unicellular ingested algae are visible in the midgut at all stages. at, apical tuft; hg, hindgut; lb, lateral bristle; lhc, lateral hooked cilia; lo, larval ocellus; mg, midgut; mt, metatroch; pyg, pygidium; st, stomodeum. Scale bars are 50 μm.

Stage L2—36–72 hours (Fig. 2b–c):
Metatrochophore
Size: 180 × 90 μm
Ciliary structures: apical tuft present; one pair of lateral bristles form; trochal band absent

Ocelli: 2; one lateral pair
References: Henry (1986) fig. 3b–c; Bonch-Bruevich and Malakhov (1987) fig. 1

By this stage gastrulation is complete, forming a tripartite gut. A ventrally opening ciliated stomodeum is visible anteriorly. The more medial stomach occupies about half the volume of the larva, and a much smaller intestine is located just anterior to the pygidium. The anus opens dorsally. The gut is functional at this time, as evidenced by algal particles in the stomach. A pair of stiff lateral hooked bristles, composed of hooked cilia, take the place of the lateral hooked cilia (Henry, 1986). A distinct trochal band is not present at this stage.

Stage L3—3–30 days (Fig. 2d–i):
Size: 180–320 × 90–180 μm
Ciliary structures: apical tuft present; mesotroch present; lateral bristles persist
Ocelli: early: 2; one lateral pair; late: 4; one lateral pair and one medial pair
References: early: Cazaux (1965) plt. 2, fig. 5; late: Cazaux (1965) plt. 3, fig. 6; Enders (1909) plt. II, fig. 9

Early period, 3–10 days. In this period the relative size of the stomach enlarges to occupy most of the larva. A more distinct pygidial papilla forms. A distinct trochal band is first visible at the level of the intestine, here referred to as a *mesotroch,* following the terminology of Okada (1957). The lateral bristles of Stage L2 persist.

In histological sections the neuropil of the cerebral ganglion is visible anterior and dorsal to the stomodeal opening (Fig. 3a, b). At this stage other neural tissues were not visible in section, although a ventral nerve network has been reported in slightly younger specimens examined with transmission electron microscopy (Bonch-Bruevich and Malakhov, 1987). The stomodeum itself has three dorsal diverticulae and opens to the stomach through a pharyngeal valve in the midposterior floor of the stomodeal cavity (Fig. 3b). The midgut endoderm consists of relatively large cells, especially at the anteroventral side, whereas the endoderm of the intestine forms a much thinner epithelium. The anus opens from the intestine dorsally, just anterior to the pygidium (not shown).

Mesoderm-lined coelomic compartments are visible anterior to the stomodeum and along the ventral midgut and hindgut (Fig. 3a, b). These observations are consistent with those of Bonch-Bruevich and Malakhov (1987), who report one unpaired preoral coelom and three pairs of trunk coeloms, although we were unable to locate with certainty the boundaries between the trunk cavities.

Identifiable cell types present at this stage include neurons, secretory digestive cells, trochoblasts, and muscle

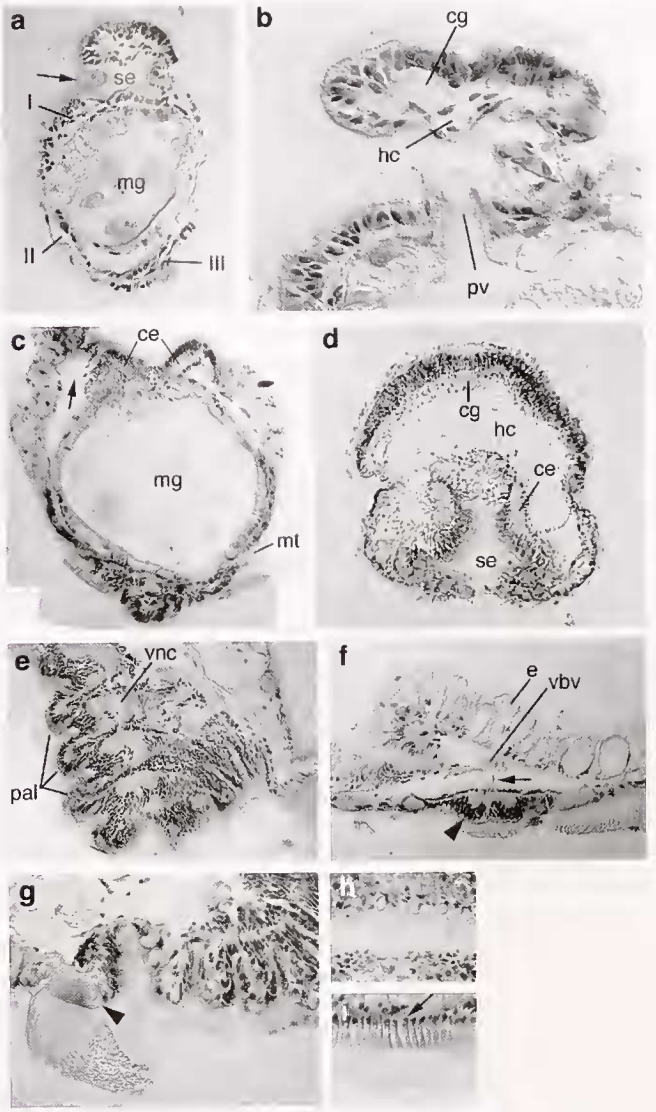

Figure 3. Semithin plastic sections of stages L3 and L4 larvae stained with hematoxylin, eosin, and alcian blue. (a) Early stage L3 larva in sagittal section; ventral is to the left and anterior toward the top of the page, unless noted. Arrow points to algal particle entering stomodeum. Roman numerals denote locations of three trunk coelomic spaces. (b) Higher magnification view of same stage larva as in (a). (c) Oblique frontal section of a late stage L3 larva. Arrow points to an anterior septum dividing one of the region A segments. (d) Oblique transverse section through head of stage L4 larva with dorsal side up. (e) Frontal section through palette rudiments (segments B2–B5). (f) High-magnification view of transverse section through ventral body wall of stage L4 larva midway between the mouth and anterior mesotroch. Arrowhead points to the ventral nerve cord. Arrow indicates the ventral mesentery. (g) Transverse section, with anterior to the left, through posterior metatroch (setiger B2) and palette rudiments (setigers B3–B5). Arrowhead points to trochal cell. (h, i) Sections through trochal bands at stage L4 tangential to body wall. Arrow points to line of basolateral trochal cell nuclei. ce, circumesophogeal connective; cg, neuropil of cerebral ganglion; e, endodermal cell of the midgut; hc, head coelom; mg, midgut; mt, mesotroch; pal, pallette rudiment; pv, pharyngeal valve; se, stomodeum; vbv, ventral blood vessel; vnc, neuropil of ventral nerve cord.

cells. Also visible are light-emitting photocytes ventrolateral to the intestine; these, described by Henry (1989), are functional by stage L2. Staining with alcian blue (not shown) reveals large mucosal cells dorsal and lateral to the stomodeum.

Late period, 11–30 days. This period is morphologically similar to the preceding, the most obvious difference being the addition of a pair of dorsomedial eyes. The stomach becomes still larger relative to the overall body, and the mesotroch widens with it. The pygidial papilla becomes longer and more distinct. The apical tuft is still present along with the lateral bristles, both of which are lost by the end of this stage. These observations correlate well with previous descriptions (Enders, 1909; Cazaux, 1965).

In situ hybridization with a *β-tubulin* riboprobe reveals cells with extensive ciliation in the apical tuft, stomodeum, and mesotroch (Fig. 4a). However, at this stage our probe does not reveal neural elements.

Stage L4—30–60 days (Fig. 5a, b):
Size: approx. mean 600 × 400 μm
Ciliary structures: apical tuft lost; two mesotrochs
Ocelli: 6; 2 pairs lateral, 1 pair medial
References: Cazaux (1965) plt. 4, figs. 7–8; Enders (1909) plt. II, fig. 10

Two major changes from stage L3 are evident in this stage. The first is the appearance of a second trochal band just anterior to the existing mesotroch. The second change is the advent of overt segmentation in the region between the posterior mesotroch and the pygidium. Three distinct annular bulges are visible in this region; as becomes evident in later stages, these are rudiments of setigers B3–B5. The segmental anlage of the anterior 11 setigers are not apparent by visual inspection, but the prospective cell populations of the parapodia are present. This was shown by staining with an anti-Distal-less antibody that recognizes the prospective apical cells of body wall outgrowths (refer to Panganiban *et al.*, 1997). The basic structure of the gut present from stage L2 persists, with the intestine occupying the postmesotrochal segmented region. A second pair of lateral ocelli develop at this stage, making a total of six ocelli in three bilateral pairs.

The central nervous system now has the basic components of the juvenile. The cerebral ganglion forms a disk just beneath the most rostral epidermis (Fig. 3d). The circumesophogeal connectives flank the stomodeum (Fig. 3c, d) and join in the ventral midline at the anterior midgut level (Fig. 3f). The ventral nerve cord (VNC) remains paired as it travels toward the posterior, with numerous commissures connecting the bilateral segmental ganglia. In the overtly segmented posterior region (setigers B3–B5), distinct paired segmental ganglia are visible (Fig. 3e). Late in this stage the two hemilateral cords of the VNC diverge anterior to the mesotrochs. This splitting of the paired nerve cords results in the adult arrangement of the CNS: laterally placed nerve cords in setigers A1–A11 join at setiger B1 and run at the ventral midline more posteriorly (Martin and Anctil, 1984). The lateral divergence of the anterior nerve cord is visible in

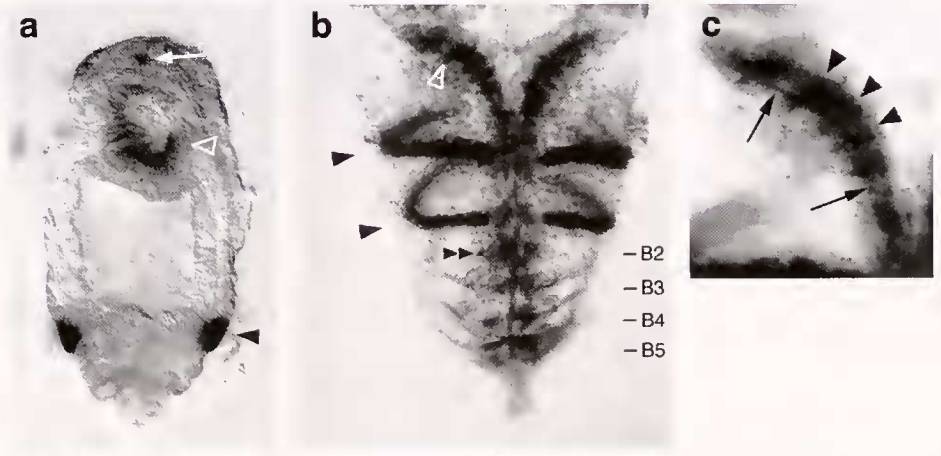

Figure 4. Whole mount *in situ* hybridization to a digoxygenin-labeled antisense *β-tubulin* riboprobe. Anterior is toward the top of the page in each view. (a) Stage L3 larva viewed from the ventral side in optical section. Staining is visible at the base of the apical tuft (arrow), around the posterior stomodeum (open arrowhead), and in the trochoblasts of the mesotroch (arrowhead). (b) Ventral view of a stage L4 larva. The dorsal body wall and head have been dissected open for photography. Strong staining is seen in the trochoblasts of both mesotrochs (arrowheads), and in the anterior (open arrowhead) and posterior (double arrowhead) ventral nerve cord. Setiger numbers of posterior ganglia are labeled on the right. (c) Higher magnification view of anterior ventral nerve cord. The axon tract of the nerve cord (arrows) is visible just subjacent to serially iterated blocks of staining ectodermal cells (arrowheads).

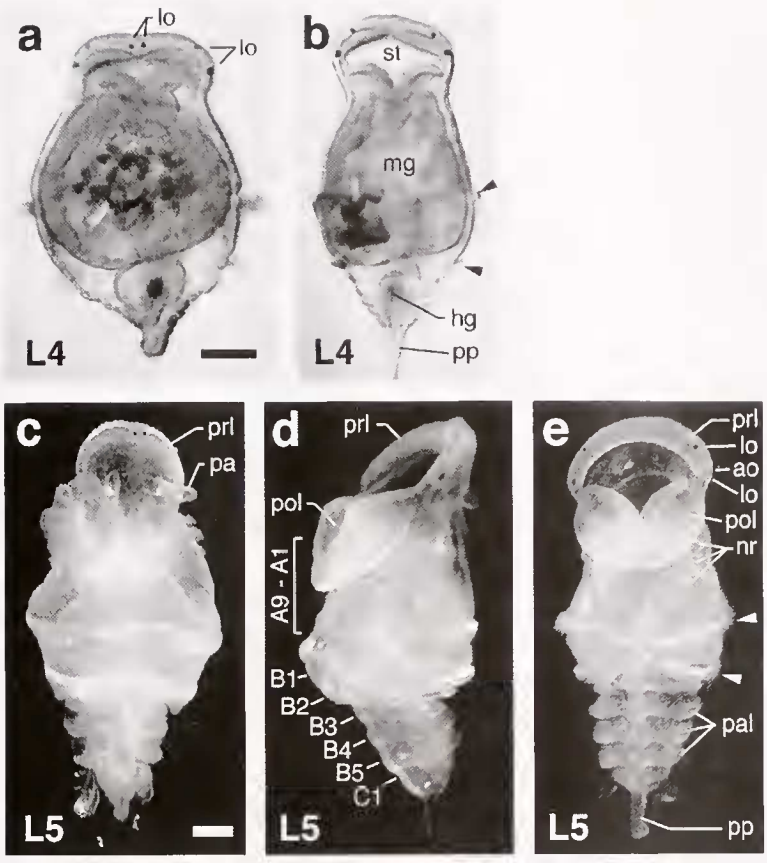

Figure 5. Larvae, stages L4 and L5. Anterior and posterior mesotrochs are marked by arrowheads. (a, b) Stage L4 larvae at 30 days in dorsal and ventral views. (c–e) Stage L5 larvae at 60 days in dorsal, lateral, and ventral views respectively. The locations of adult setiger rudiments are labeled in (d). Note the appearance of the red adult ocelli, visible in (e) along with the persistent larval ocelli. ao, adult ocellus; hg, hindgut; lo, larval ocellus; mg, midgut; nr, notopodial rudiment of setigers A1–A9; pa, palp rudiment; pal, palette rudiment of setigers B3–B5; pol, post-oral lobe; pp, papilla; prl, pre-oral lobe; st, stomodeum. Scale bars are 100 μm.

the expression pattern of *β-tubulin* visualized by *in situ* hybridization (Fig. 4b, c). The fact that *in situ* hybridization to *β-tubulin* transcripts failed to detect a distinct VNC prior to this stage suggests that the VNC had not yet formed.

Segmental boundaries are not distinguishable, by conventional microscopy, anterior to the mesotrochs in any tissue. However, *in situ* hybridization to *β-tubulin* transcripts reveals that reiterated ganglionic cell populations, presumably segmental, are present at this stage (Fig. 4b, c). Bilateral ganglionic cell populations are also visible in the overtly segmented anlagen of setigers B2–B5 (Fig. 4b).

Capacious coelomic cavities with distinct septa surround the larval foregut, as seen in frontal section (Fig. 3c). Transverse sections at the middle of the larva reveal bilateral coelomic cavities, separated by a ventral mesentery, medial to the nerve cord. Between these cavities and the gut, the ventral blood vessel is located at the midline (Fig. 3f).

A particularly distinctive cell type is that of the ciliated cells of the trochal bands. These are large prismatic cells with a uniform granular cytoplasm (Fig. 3g). They are extended along the anterior-posterior axis, and packed regularly in a continuous circumferential ring (Fig. 3h). The cell nuclei are positioned at the basilateral ends of the cells (Fig. 3i).

Stage L5—approx. 60 days (Fig. 5c–e):
Competent to metamorphose
Size: approx. mean 800 × 400 μm
Ciliary structures: apical tuft absent; two mesotrochs
Ocelli: 8; 4 lateral, 2 medial, 2 lateral adult ocelli
References: Cazaux (1965) plt. 5, fig. 10; Enders (1909) plt. II, figs. 11–12

At this stage larvae are competent to metamorphose—in fact, we observed one specimen from this stage that had reached late stage L7 within 6 h after transfer from mass culture to a petri dish with fresh seawater. Specimens from this stage routinely passed completely through metamor-

phosis overnight, indicating that the rudiments of all juvenile structures are present.

As compared with stage L4, the postoral lobe grows disproportionately with respect to the preoral lobe and folds towards the posterior. Many eosin-reactive secretory cells are visible in section in the epidermis of this organ (Fig. 6b). Appearing at this stage are visible palp and anterior parapodial rudiments. A pair of red adult ocelli appear at the most lateral margin of the preoral lobe. The smaller dark larval ocelli remain throughout the stage. In section, the setal sacs and septation of segments A1–A9 are apparent (Fig. 6a, b). The epidermis in the region of prospective setigers B2–B5 develops deeper infolding, creating distinct annuli anchored at the ventral midline (Figs. 3g, 6b). However, the region around the two mesotrochs has yet to exhibit any segmental character visible either in the exterior morphology or in section. A pair of lateral outgrowths emerge just anterior to the pygidium, which Enders (1909) identifies as the notopodia of segment C1. These bear stout setal sacs (not shown). Based on the locations of ganglia in the B and C regions and the developing parapodia of the A region, it is possible to locate the primordia of the first 15 adult setigers at this stage (Fig. 5d). The identity of the posterior mesotroch with the aliform notopodia of setiger B1 can be inferred from Hox gene expression patterns (Irvine, 1998; Irvine and Martindale, 1999b). Ironically, the longest adult segment, B2, forms from the shortest, most cryptic of the larval setigers.

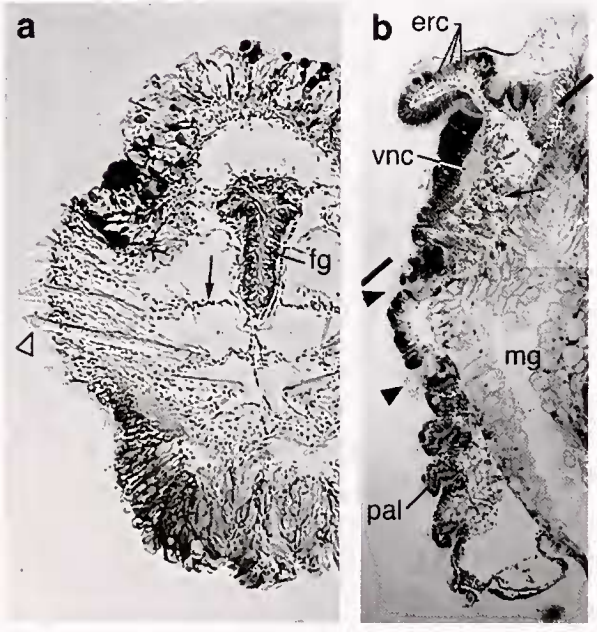

Figure 6. Semithin sections of stage L5 larvae stained as in Figure 7. (a) Oblique transverse section through region A. Dorsal is towards the top of the page. Open arrowhead points to a typical anterior seta. Arrow points to an anterior septum. (b) Parasagittal section with ventral to the left and anterior up, cutting through several anterior setal sacs (arrow) and both mesotrochs (arrowheads). Bars denote approximate plane of section shown in (a). erc, eosin-reactive cells; fg, foregut; mg, midgut; pal, palette rudiment of setiger B2; vnc, axon tract of ventral nerve cord.

The hemilateral cords of the anterior ventral nerve cord have continued to diverge from the ventral midline to approach the ladderlike form of the adult nervous system in setigers A1–A9 (Martin and Anctil, 1984). The basic structure of the nervous system more posteriorly persists, as described for stage L4.

Stage L6—approx. 60 days (Fig. 7a–d):
Mid-metamorphosis
Size: 1–2 mm
Ciliary structures: apical tuft absent; two mesotrochs
Ocelli: 8; 4 lateral, 2 medial, 2 lateral adult ocelli
References: Cazaux (1965) plt. 5, fig. 10; Enders (1909) plt. 11, figs. 11–12

This transitory stage is characterized by the transformation of larval to adult structures (Fig. 1). The prostomium and peristomium form by the retraction of the preoral lobe and the folding rostrally of the postoral lobe. The pair of dorsomedial ocelli disappear during this stage (compare Fig. 7a and 7c). The two larval pairs of lateral ocelli persist, with the adult ocelli roughly coincident with the most ventrolateral pair of larval ocelli. The parapodial rudiments of setigers A1–A9 emerge laterally, correlated with a dorsoventral flattening of region A. The anterior mesotroch degenerates, and the posterior mesotroch becomes incorporated into the aliform notopodia of segment B1, which appear dorsolaterally. This fate is confirmed by *in situ* hybridization to Hox segmental markers (Irvine, 1998; Irvine and Martindale, 1999b). Just caudally, the digestive and accessory feeding organs of setiger B2 appear along the dorsal surface with a swelling of this portion of the larva. The three annular bulges evident at stage L4 expand to take on the shape of the palettes of segments B3–B5. The notopodia of segment C1 continue to project ventrolaterally.

Stage L7—approx. 60 days (Fig. 8a–b):
Juvenile
Size: 2–3 mm
Ocelli: 2 lateral adult ocelli (2 pairs of larval ocelli degenerate)
References: Cazaux (1965) plt. 6, figs. 11–14

At the completion of metamorphosis, the juvenile worm has taken on the general form of the adult for the head and anterior 15 setigers. The most conspicuous change from stage L6 is the extreme extension of the body axis from setigers B1–B5. In addition, the aliform notopodia in setiger B2, the accessory feeding organ in setiger B2, and the palettes in setigers B3–B5 all extend out from the body wall and assume their adult form. The remaining two pairs of larval ocelli degenerate, leaving the larger red adult ocelli. The remainder of the roughly 40 abdominal gametogenic

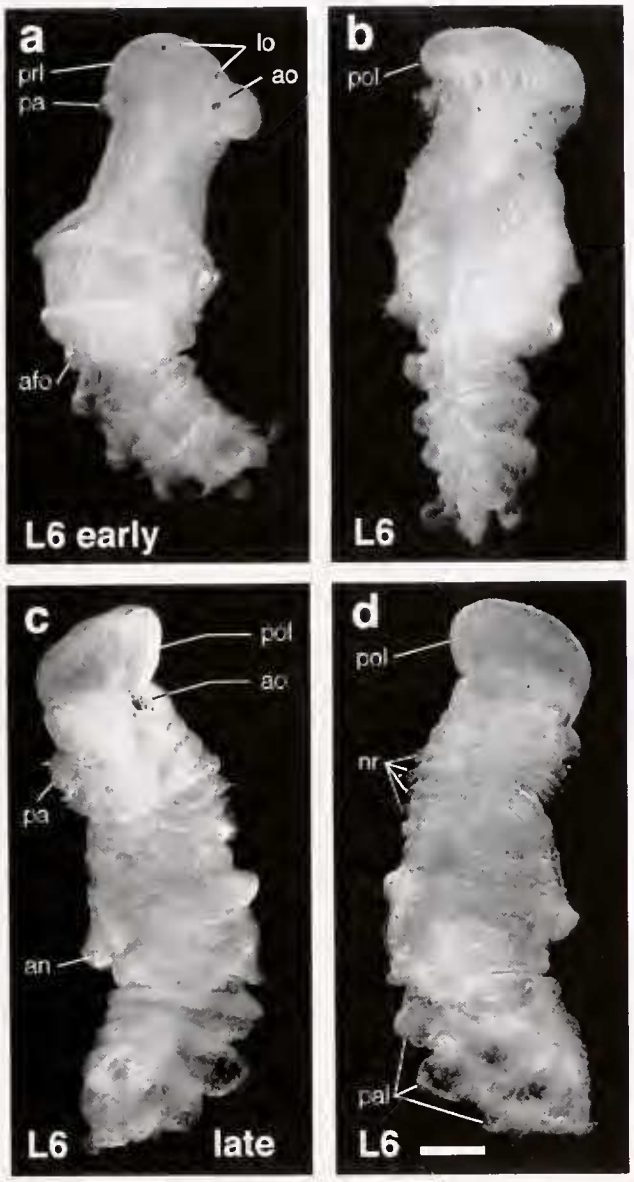

Figure 7. Stage L6 larvae. (a, b) Early stage L6 larva in dorsolateral and ventrolateral views. (c, d) Late stage L6 larva in dorsolateral and ventrolateral views. Note the rotation of the postoral lobe to an anterior-facing direction in comparison with the orientation at stage L5. afo, accessory feeding organ rudiment; an, aliform notopodium rudiment; ao, adult ocellus; lo, larval ocellus; nr, notopodial rudiment of setigers A1–A9; pa, palp rudiment; pal, palette rudiment of setigers B3–B5; pol, postoral lobe; prl, preoral lobe. Scale bar is 100 μm.

segments have yet to be produced. This occurs by interpolation between setiger C1 and the pygidium (Cazaux, 1965).

Tube construction was never observed in these cultures. This is probably because that substrate was never provided, the stage L7 juveniles always being kept in glass or plastic vessels without mud or sand (Irvine and Martindale, 1999a).

Discussion

Developmental variation within the genus Chaetopterus

The genus *Chaetopterus* has several species that show variation in overall adult size, tube morphology, and details of parapodial and setal form. Published descriptions of *Chaetopterus* development differ from our results in some respects. Cazaux's (1965) figure 5 is a drawing of a 48-h larva corresponding in part to our observations. However, our cultures and those described in Henry (1986) do not reach the general morphology depicted until at least 72 h, despite higher culture temperatures. Four other differences are apparent between our results and those in figure 5 of Cazaux (1965): (i) we do not detect trochal bands around the stomodeum; (ii) a distinct intestine is visible, rather than the extension of the posterior stomach shown; (iii) the mesotroch is more posterior in our preparations; (iv) only one pair of laterally placed eyes are visible rather than the two pairs depicted. Since Cazaux's specimens came from the Atlantic coast of France, he may have been observing another species of *Chaetopterus*—neither *C. variopedatus* Renier, 1804, nor *C. variopedatus sensu* Enders, 1909, but possibly *C. valencinii* Quatrefages, 1866 (M. E. Petersen, Copenhagen Museum, pers. comm).

Trochal bands

As mentioned, a circumferential ciliary band appears midway along the anterior-posterior body axis at Stage L3; following Okada (1957), we have termed this band a mesotroch. At Stage L4 another trochal band, which we also call a mesotroch, forms just anterior to the first band. Rouse (1999) characterizes this younger band as a metatroch, following early work of Wilson (1882) that depicts a larva resembling our Stage L3. However, tracing the fate of both these trochal bands ahead in ontogeny reveals that they come to lie well within the segmented trunk of the larva, contrary to the definition of a metatroch as a presegmental structure lying on the peristomium (Rouse, 1999). Thus, rather than a metatroch having evolved within the Chaetopteridae lineage, as concluded in the transformations of the Rouse (1999) analysis, a different type of trochal band arose, not strictly homologous to a metatroch.

Although the *Chaetopterus* mesotrochal bands are not metatrochs, as defined above, they may be homologous to other types of trochal bands at the level of their developmental pathway. Rouse's (1999) analysis indicates that the various types of larval trochal bands can appear and be lost independently in different lineages; *i.e.*, as characters they exhibit a high degree of homoplasy. This finding suggests that trochal bands share a developmental pathway that can be activated at various levels along the anterior-posterior axis.

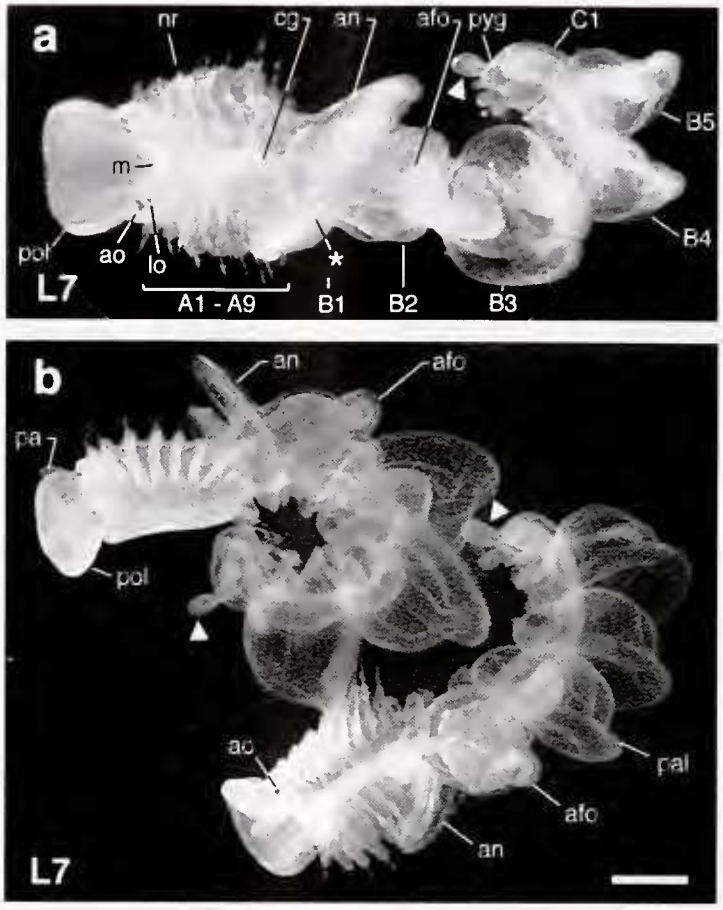

Figure 8. Juvenile worms within 1 day of metamorphosis at about 60 days. Anterior is to the left. (a) Dorsal view. Letters and numbers indicate adult setiger locations. Asterisk denotes the ciliary groove of the aliform notopodium of setiger B1, derived from the posterior mesotroch. Arrowhead points to a parapodium of the first 'abdominal' setiger C1. (b) Two newly metamorphosed specimens. Upper specimen is in ventrolateral view and lower is in dorsolateral view. Arrowheads as in (a) above. afo, accessory feeding organ rudiment; an, aliform notopodium rudiment; ao, adult ocellus; cg, ciliated groove; lo, larval ocellus; m, mouth; nr, notopodial rudiment of setigers A1–A9; pa, palp rudiment; pal, palette rudiment of setigers B3–B5; pol, postoral lobe; pyg, pygidium. Scale bar is 100 μm.

Larval segmentation and relationship to adult body plan

The most commonly described form of larval development in polychaetes is the production of a trochophore larva that adds segments sequentially from a posterior growth zone to produce a nectochaete larva (Okada, 1957; Anderson, 1966). There is some controversy over whether the first three larval segments develop in the same sequential manner as subsequent segments, but in typical cases the demarcation of each of the segmental boundaries is evident in the external form of the larva from a very early stage. *Chaetopterus* represents a distinct departure from this general pattern. The first external signs of segmentation are the rudiments of segments B3–B5 visible at stage L4, at an age of 30 days. At no point does the metatrochophore take on the overtly segmented form of the typical nectochaete larva. However, some incipient segmentation is present before it becomes visible externally. Bonch-Bruevich and Malakhov (1987) describe three trunk coeloms existing at stage L2, which is consistent with our stage L3 sections (Fig. 3a). If we use the trochal bands as landmarks, the anterior trunk coelom ('I' in Fig. 1 stage L3) roughly corresponds with the position of adult segments A1–A9, the middle coelom ('II') with segment B1 or segments B1 and B2, and the posterior coelom ('III') with juvenile segments B3–B5. By stage L4 the segmental character of the ventral nerve ganglia is apparent in *β-tubulin* expression, even though no segmental divisions are visible by conventional microscopy (Fig. 4b, c). Expression of Distal-less protein also reveals segmentally iterated structures, the parapodia of setigers A1–A9, at stage L4, before they are evident morphologically (Panga-

niban *et al.*, 1997). Our observations never give the impression that these segmental rudiments are produced sequentially, anterior to posterior, from a mesodermal band or bands.

We propose the hypothesis, supported by all the existing data from this and other studies, that the anterior 15 segments in *Chaetopterus* are formed by subdivision of existing anlage, rather than by sequential addition from a growth zone. The segments B1–B5 are formed first, at stage L4, by subdivision of the stage L3 coeloms II and III. Segments A1–A9 form later, at stage L5, by subdivision of the stage L3 coelom I. This hypothesis does not rule out the production of the segmented body elements from a teloblastic growth zone, but does temporally dissociate the production of those elements from their morphogenesis as discrete segmental structures. Other recent work has shown that Hox gene expression begins as early as stage L2 in the putative growth zone (Irvine, 1998; Irvine and Martindale, 1999b; Kevin J. Peterson, pers. comm.). If the Hox genes are acting as segmental specification genes at this stage, this early onset of expression suggests that the delay in the appearance of overt segmentation in *Chaetopterus* is the result of a lengthening of the period between molecular specification of segments and their morphogenesis. Testing the accuracy of this model will require more extensive analysis of intervening stages, possibly using histological sections or cell-labeling techniques.

Even if the correlation of early larval structures with their final segmental products differs from what we have proposed, the timing and nature of segmental differentiation in *Chaetopterus* remain highly diverged relative to the patterns seen in related polychaete families. This is not surprising given the extreme level of adult body plan divergence. What is remarkable is how far back into larval development the changes in segment formation extend.

Phylogenetic position and larval evolution

A consideration of the phylogenetic position of *Chaetopterus* within the Polychaeta can give some insight into the probable ancestral larval form and the evolutionary changes that must have taken place to result in the animal described here. The family Chaetopteridae has generally been allied to the spionid families (Dales, 1962; Fauchald, 1977), to the sabellids, or to both (Fitzhugh, 1989). A cladistic analysis of polychaete relationships by Rouse and Fauchald (1997) presents two alternatives for the position of the Chaetopteridae, depending on the method used to code character states. In the first case (Fig. 9a), which uses presence/absence coding, initial weighting of characters by their dependence on other characters, and sequential weighting based on consistency index, the Chaetopteridae are a sister group to the traditional spionid families (Spionidae, Apistobranchidae, Trochochaetidae, Longosomatidae, Magelonidae, and Poecilochaetidae). This is the topology favored by Rouse and Fauchald, who include the Chaetopteridae in a clade called the Spionida (figs. 70 and 73 in Rouse and Fauchald, 1997). (Examination of other members of the Chaetopteridae, such as *Spiochaetopterus* [Bhaud *et al.*, 1994], makes the spionid connection very clear.) On the other hand, when multi-state characters are used (fig. 71 in Rouse and Fauchald, 1997), the parsimony analysis results in the Chaetopteridae being a sister group to the sabellid families and pogonophorans (Frenulata, Vestimentifera, Sabellariidae, Sabellidae, and Serpulidae; collec-

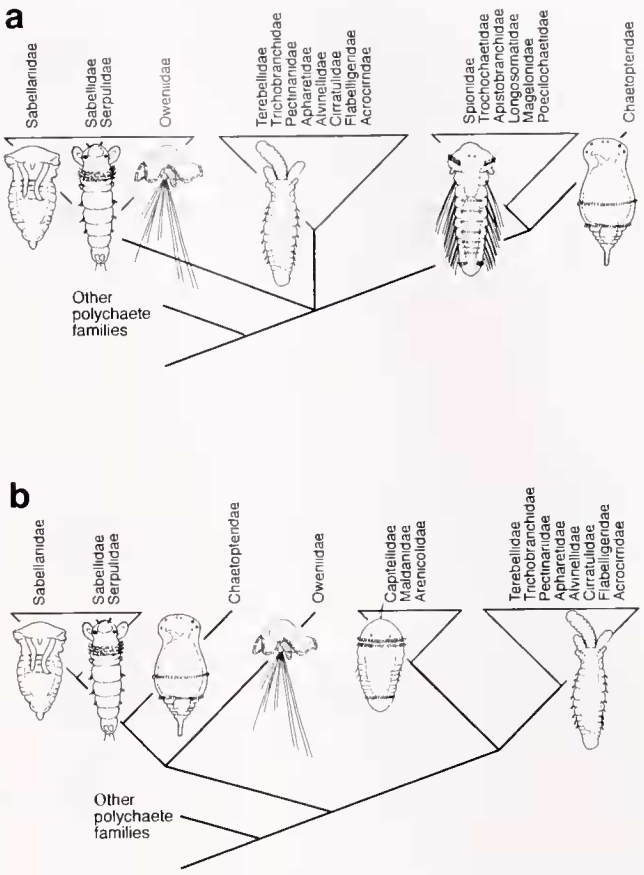

Figure 9. Two alternative cladograms showing larval form of *Chaetopterus* compared with that of related polychaete families. The cladograms are adapted from Rouse and Fauchald (1997)—(a) from figure 70 and (b) from figure 71. The presence of the Pogonophora in the sabellid clade is omitted from these diagrams. Note that in either cladogram, outgroup taxa to the Chaetopteridae have overtly segmented early larvae, indicating that the chaetopterid larval form and ontogenetic heterochrony are unique to that lineage. Representative larval forms are adapted from the following sources: spionid, *Polydora webster* (Blake, 1969); terebellid, *Ramex californiensis* (Blake, 1991); oweniid, *Owenia fusiformis* (Plate and Husemann, 1997); sabellid, *Megalomma vesiculosum* (Wilson, 1936); sabellariid, *Lygdamis muratis* (Bhaud and Cazaux, 1987); capitellid, *Capitella capitata* (Plate and Husemann, 1997).

tively called the Sabellida) (Fig. 9b). Using weighted presence/absence coding but adding larval characters to the data, a more recent analysis (Rouse, 1999) also allies the Chaetopteridae with an order Sabellida.

In the first case, depicted in Figure 9a, overtly segmented early larvae are common both in the sister group of the chaetopterids, the spionid clade, and in the outgroups, the terebellid and sabellid clades. With the exception of the oweniids, the larvae can be described as variants of a basic nectochaete type (Okada, 1957; Wilson, 1948). Thus, by parsimony, the hypothetical common ancestor of the entire clade depicted would have some type of nectochaete larva. The divergent larval forms of *Owenia* and the Chaetopteridae are autapomorphies of their families in this scheme. Other chaetopterid genera share the basic larval form of *Chaetopterus* (Bhaud and Cazaux, 1987). Within the family there are no larvae with morphology intermediate to a nectochaete type, indicating that the ancestor of extant family members had already developed this modified larval ontogeny.

In the second case, depicted in Fig. 9b, the Chaetopteridae are a sister group to a sabellid clade, and these groups together are a sister group to the Oweniidae. This entire clade is in turn a sister group to a clade consisting of the capitellid and terebellid families. Once again, because of the phylogenetically widespread presence of overtly segmented nectochaete larvae in sister groups and outgroups, the hypothetical common ancestor at the basal node of this cladogram would be some sort of nectochaete.

From the foregoing it follows that, regardless of the phylogenetic scheme favored, the larval ontogeny of *Chaetopterus* is highly modified from a probable nectochaete ancestor. This ancestor developed as an initial trochophore larva forming three larval segments by subdivision of mesodermal bands. Subsequent segments were added sequentially from a pre-pygidial growth zone. In the chaetopterid lineage the following evolutionary changes in ontogeny took place: (1) loss of early body wall segmentation; (2) loss of larval setae; (3) loss of prototroch; (4) gain of one or more mesotrochs; (5) delay in segmentation of anterior trunk; (6) modification of parapodia in region B setigers to form specialized feeding and pumping organs.

Ontogenetic heterochrony

As described above, the temporal pattern of segmentation in *Chaetopterus* is modified from that typical of annelids. This pattern correlates with the regionalization of the adult body plan along the anterior-posterior axis. In the common annelid form, segmental morphology is homonomous, and segments form in a strict anterior-posterior temporal sequence. In many groups, there is a measure of heteronomy in segment form, such as groups of segments bearing branchiae in spionids, or nereidids with differing anterior and posterior parapodial morphology. However, even where this regionalization of adult body plan exists, the segments develop sequentially in the larva. In *Chaetopterus*, on the other hand, each tagma, or body region, develops overt segmentation at a different time. The first segments clearly visible are those of setigers B1–B5 at stage L4; these form the middle tagma of the adult body. Segmentation more anterior, which would be morphologically apparent in a nectochaete larva, is visible at this stage only by the use of molecular markers, such as *β-tubulin* and Distal-less, to show segmental cell populations.

These changes in the temporal pattern of development from the ancestral state can be regarded as ontogenetic heterochrony in an evolutionary sense. In deBeer's terminology (DeBeer, 1958), the structures of setigers B3–B5 exhibit *acceleration* relative to the anterior setigers, while all segments exhibit *deviation* in morphogenesis relative to the ancestral form.

An important question raised by consideration of this case of heterochrony is whether the changes in larval ontogeny in the extant chaetopterids were part of the direct evolutionary transformation leading to the tagmatization of the adult body plan, or if they are secondary modifications of larval development independent of the changes in adult body plan. In the first case, the changes in ontogeny would have to result in the changes in the adult without significant deleterious effect on larval survival, and thus overall fitness. In the second case, it could be that extinct ancestors of *Chaetopterus* evolved the heteronomous body form seen today using the primitive larval developmental program based on sequential anterior-posterior segmentation. After these changes produced the tagmatization of the adult body, the heterochrony in larval ontogeny may have evolved independently either as adaptations to larval ecology or as changes in developmental pathways that may or may not have adaptive value. Further comparative work on the molecular basis of polychaete development may help to distinguish between these scenarios.

Acknowledgments

The authors thank Mary E. Petersen of Copenhagen Museum for extensive discussion and information on chaetopterid systematics. Jonathan Q. Henry was a great help sharing his expertise on rearing larvae, and he, Susan D. Hill, Elaine Seaver, and Michael LaBarbera provided insightful commentaries on earlier versions of the manuscript. S. Q. I. was supported by National Institutes of Health training grant T32HD07136-20, National Science Foundation dissertation improvement grant 9623453, and a Hind's Fund grant from the University of Chicago. M. Q. M. was supported by National Science Foundation grant 9315653.

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